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Cellular Inhibitors of Apoptosis Proteins cIAP1 and cIAP2 Are Required for Efficient Caspase-1 Activation by the Inflammasome

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SUMMARY

Pathogen and danger recognition by the inflammasome activates inflammatory caspases that mediate inflammation and cell death. The cellular inhibitor of apoptosis proteins (cIAPs) function in apoptosis and innate immunity, but their role in modulating the inflammasome and the inflammatory caspases is unknown. Here we report that the cIAPs are critical effectors of the inflammasome and are required for efficient caspase-1 activation. cIAP1, cIAP2, and the adaptor protein TRAF2 interacted with caspase-1-containing complexes and mediated the activating nondegradative K63-linked polyubiquitination of caspase-1. Deficiency in cIAP1 (encoded by *Birc2*) or cIAP2 (*Birc3*) impaired caspase-1 activation after spontaneous or agonist-induced inflammasome assembly, and *Birc2*$^{-/-}$ or *Birc3*$^{-/-}$ mice or mice administered with an IAP antagonist had a dampened response to inflammasome agonists and were resistant to peritonitis. Our results describe a role for the cIAPs in innate immunity and further demonstrate the evolutionary conservation between cell death and inflammation mechanisms.

INTRODUCTION

The innate immune system is critical for host defense against invading microorganisms and the repair of damaged tissue. One of its most primitive responses is the elimination of compromised cells by programmed cell death, a response found in all metazoan phyla (Zitvogel and Kroemer, 2008). Among the innate immunity effectors are evolutionarily conserved germline-encoded pattern recognition receptors (PRRs) that recognize specific microbial motifs or endogenous danger signals (microbe- or danger-associated molecular patterns [MAMPs or DAMPs]). PRRs include, among others, trans-membrane Toll-like receptors (TLRs) and cytosolic nucleotide-binding domain and leucine-rich repeat-containing proteins (NLRs) which, when engaged, trigger inflammation and, in some contexts, cell death (Kumagai and Akira, 2010).

It is becoming increasingly appreciated that the pathways that initiate and regulate inflammation and innate immunity are coupled to the cell death machinery. For instance, caspases mediate key events in both types of responses and their mechanisms of activation are strikingly conserved (Yeretssian et al., 2008).

Caspase-1 plays a paramount role in inflammatory and infectious diseases. Active caspase-1 converts pro-interleukin-1β (pro-IL-1β) and pro-IL-18 into their mature cytokine forms and targets a number of other cellular proteins to amplify the inflammatory response and induce pyroptosis (Shao et al., 2007; Labbé and Saleh, 2008). Consistently, caspase-1-deficient animals are susceptible to infections because of impaired inflammation and pyroptosis (McIntire et al., 2009) and are more sensitive to the induction of colitis and colitis-associated colorectal cancer because of an inherent defect in tissue repair (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Zaki et al., 2010). In contrast, lack of caspase-1 activity is protective in diseases of excessive inflammation such as septic shock (Li et al., 1995).

A number of inflammasomes have been identified including complexes scaffolded by NLRP proteins, NAIP receptors in conjunction with NLRC4, RIG-I, and AIM2. These platforms recruit caspase-1 directly or through the adaptor protein ASC (Kofoed and Vance, 2011; Schroder and Tschopp, 2010; Zhao et al., 2011). With the exception of NLRP3 that is stimulated by a variety of disparate signals, the inflammasomes have defined agonists and primarily play a role in the detection of pathogens. However, the molecular events leading to efficient caspase-1 activation and regulation are unknown.

The inhibitors of apoptosis proteins (IAPs) are critical regulators of cell death and inflammation (Lopez and Meier, 2010; O'Riordan et al., 2008). XIAP, cIAP1, and cIAP2 consist of three N-terminal baculovirus IAP repeat (BIR) domains and a C-terminal RING domain that confers E3 ubiquitin ligase activity (Lorick et al., 1999). cIAP1 and cIAP2 also contain a central caspase-recruitment domain (CARD) involved in autoinhibition of their E3 ligase activity (Lopez et al., 2011). Insights into the function of cIAP1 and cIAP2 came with the development of synthetic IAP antagonists based on the structure of SMAC (second mitochondrial activator of caspases), an endogenous IAP inhibitor sequestered to the mitochondria but released after mitochondrial outer membrane permeabilization (MOMP). It has been shown that these SMAC mimetics (SM) selectively

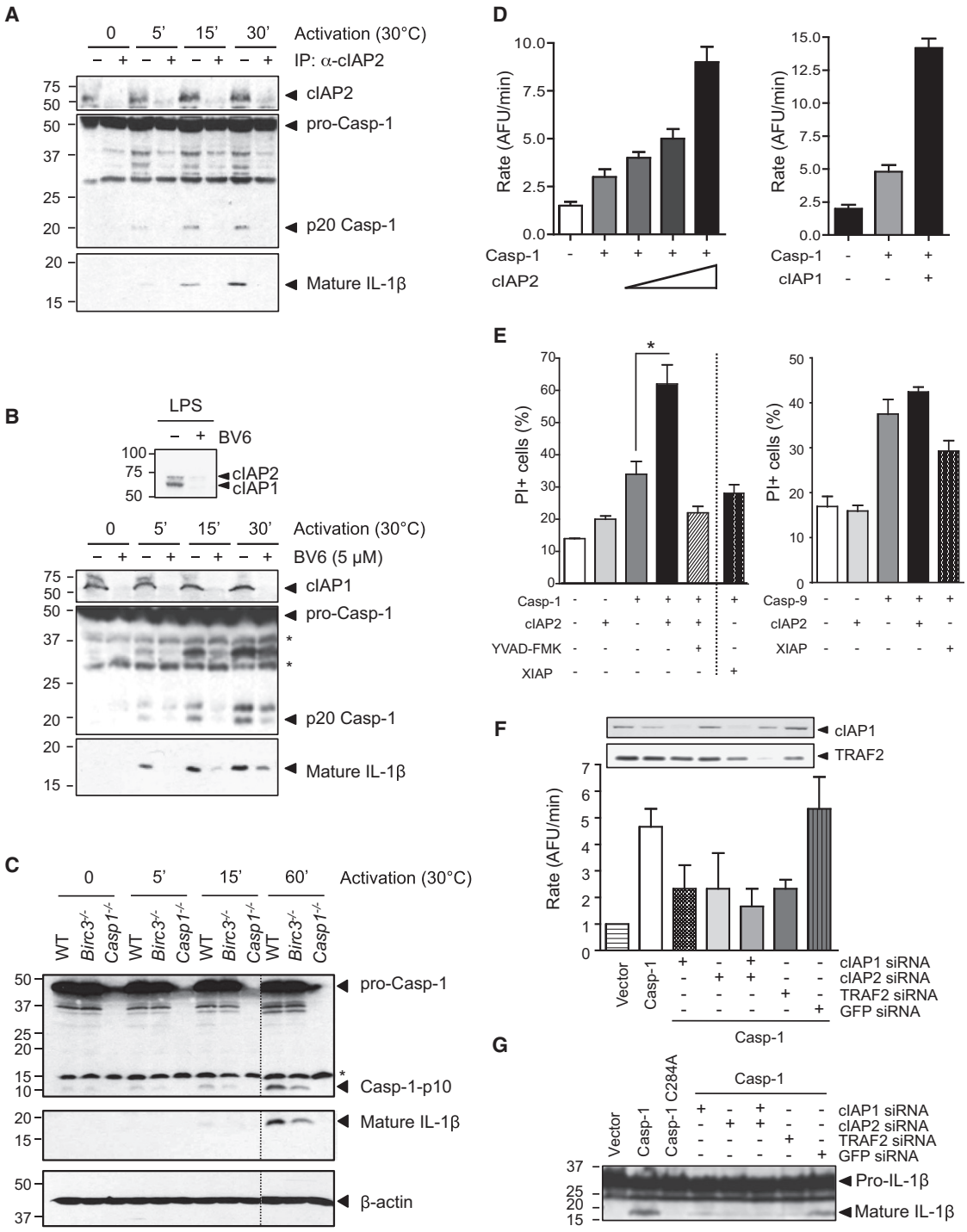

Figure 1. The cIAPs Are Required for Inflammsome Assembly and Caspase-1 Activation

(A) The cytosolic fractions of LPS-stimulated THP-1 cells were immunodepleted with cIAP2 antibodies and inflammasome assembly was induced by heating the supernatants to 30°C for the indicated time points. Caspase-1 cleavage and IL-1β processing were detected by immunoblot.

(B) The cytosolic fractions of LPS-stimulated THP-1 cells that were pretreated for 1 hr with BV6 or DMSO were heated at 30°C for the indicated time points.

(C) The cytosolic fractions of LPS-stimulated primary BMDM from wild-type (WT), *Birc3*$^{-/-}$, or *Casp1*$^{-/-}$ mice were heated at 30°C for the indicated time points.

(D) Cleavage of the caspase-1-selective substrate Ac-WEHD-AFC by lysates from HEK293T cells transfected with caspase-1 together with a vector control or increasing concentrations of cIAP2 (left) or cIAP1 (right).

(E) Propidium-iodide staining of HEK293T cells expressing caspase-1 or caspase-9 with a vector control, cIAP2, or XIAP. Where indicated, cells were treated with the caspase-1 inhibitor YVAD-FMK (50 μM).

interact with cIAP1 and cIAP2 to rapidly induce their autoubiquitination and proteasomal degradation (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). Use of SM on cancer cell lines revealed that cIAP1 and cIAP2 were responsible for K63-linked polyubiquitination of receptor interacting protein 1 (RIP1) downstream of TNFR1, promoting NF-κB activation and prosurvival signaling, while suppressing apoptosis (Bertrand et al., 2008). cIAP1 and cIAP2 also direct the degradative (K48-linked) ubiquitination of NF-κB-inducing kinase (NIK) to maintain the noncanonical NF-κB pathway in check (Varfolomeev et al., 2007; Vince et al., 2007). Degradation of cIAP1 and cIAP2 by SM thus results in spontaneous activation of this pathway and overproduction of TNF-α, which induces apoptosis in an autocrine loop.

In addition to promoting cell survival, the IAPs exert important roles in inflammation and innate immunity. Indeed, cIAP2-deficient mice have reduced cytokine amounts in response to LPS and are resistant to LPS endotoxemia (Conte et al., 2006). Furthermore, *Xiap*$^{-/-}$ mice have an impaired innate immune control of *Listeria monocytogenes* infection (Bauler et al., 2008). We have recently identified cIAP1 and cIAP2 as important effectors of innate immunity, mediating critical steps in NOD signaling (Bertrand et al., 2009). Moreover, cIAP1 and cIAP2 were shown to mediate MAPK activation downstream of TLR4 signaling by catalyzing degradative K48-linked ubiquitination of TRAF3 (Tseng et al., 2010).

Although cIAP1 and cIAP2 have been implicated in key processes regulating apoptotic caspases and innate immunity, their roles in modulating the inflammatory caspases and inflammasome pathway are unclear. Here we report a function of cIAP1 and cIAP2 in stimulating caspase-1 activation and inflammasome-dependent innate immunity signaling. cIAP1 and cIAP2 interacted with caspase-1 through their respective N termini in complexes containing TRAF2 and directed nondegradative K63-linked polyubiquitination of caspase-1. We showed that depletion of cIAP1 or cIAP2 or deletion of *Birc2* (encoding cIAP1) or *Birc3* (encoding cIAP2) markedly suppressed inflammasome activation. Macrophages from *Birc3*$^{-/-}$ mice were deficient in caspase-1 activation and subsequent production of IL-1β in response to various inflammasome agonists. In addition, *Birc2*$^{-/-}$ or *Birc3*$^{-/-}$ mice, or wild-type animals treated with SM, had dampened neutrophil infiltration and cytokine production in response to exposure to inflammasome stimuli. Thus, our results define a role of cIAP1 and cIAP2 in inflammation and innate immunity through the molecular regulation of the inflammasome.

RESULTS

cIAP1 and cIAP2 Are Required for Inflammasome Assembly and Caspase-1 Activation

We initially investigated whether cIAP1 and cIAP2 are important for caspase-1 activation, by using an established cell-free system in which the inflammasome is spontaneously activated (Martinon et al., 2002). This approach allowed us to study the role of the cIAPs in the absence of any external agonists, overexpression systems, or cell death signals. As expected, incubation of lysates from differentiated THP-1 monocytes at 30°C resulted in rapid inflammasome formation and caspase-1 activation, as determined by the appearance of the p20 subunit of the active enzyme and the concurrent processing of pro-IL-1β into its mature p17 fragment (Figure 1A). Immunodepletion of cIAP2 (Figure 1A) or treatment of cells with the SM BV6, which rapidly triggers the degradation of both cIAP1 and cIAP2 but inefficiently depletes XIAP (Figure 1B, top; Varfolomeev et al., 2007), markedly inhibited caspase-1 activation and IL-1β processing (Figures 1A and 1B, bottom). To rule out any indirect effects of acute cIAP depletion, we examined spontaneous inflammasome activation in lysates of bone marrow-derived macrophages (BMDM) isolated from wild-type, *Casp1*$^{-/-}$, or *Birc3*$^{-/-}$ mice. Activation of caspase-1 and production of mature IL-1β were observed within 15 min of inflammasome stimulation in wild-type cell lysates, but were markedly dampened in lysates from *Birc3*$^{-/-}$ cells (Figure 1C). Consistent with these findings, overexpression of cIAP1 or cIAP2 in HEK293T cells markedly enhanced caspase-1 catalysis (Figure 1D) and caspase-1-dependent cell death (Figure 1E). This stimulatory function on caspase-1 was not shared by caspase-9, an apoptosis initiator caspase, and was specific to cIAP1 and cIAP2, as shown by the fact that expression of another IAP family member, XIAP, had no effect on caspase-1-mediated cell death (Figure 1E). Concordantly, siRNA-mediated depletion of endogenous cIAP1 or cIAP2, alone or in combination, dramatically blunted caspase-1 activity toward its selective fluorogenic tetrapeptide substrate WEHD-AFC (Figure 1F) or its natural substrate pro-IL-1β (Figure 1G). Depletion of TRAF2, an adaptor protein constitutively associated with the cIAPs (Rothe et al., 1995; Shu et al., 1996), also inhibited caspase-1 enzymatic activity (Figures 1F and 1G), suggesting that the cIAPs in association with TRAF2 exert important regulatory activity on caspase-1 activation within the inflammasome.

The cIAPs Physically Interact with Caspase-1

Our results indicating a critical role of the cIAPs in inflammasome activation suggested that cIAP1 and cIAP2 probably functioned at the level of caspase-1. To explore this hypothesis, we performed coimmunoprecipitation experiments in HEK293T cells expressing caspase-1 with or without cIAP1, cIAP2, or TRAF2. Figure 2A and Figure S1 (available online) show that cIAP1, cIAP2, and TRAF2 coimmunoprecipitated with full-length caspase-1-containing complexes. To map the domains mediating this interaction, we generated deletion mutants and examined their association by coimmunprecipitation. Deletion of the caspase-1 CARD inhibited its interaction with cIAP2-containing complexes, whereas a CARD-only protein was capable of

(F) Cleavage of Ac-WEHD-AFC by lysates of HEK293T cells transfected with siRNAs targeting cIAP1, cIAP2, TRAF2, or GFP and subsequently transfected with caspase-1. Top panels show cIAP1 and TRAF2 expression levels as detected by immunoblot.

(G) Cleavage of in-vitro-transcribed and -translated IL-1β by HEK293T lysates from cells treated as in (F). Cell-free assays were performed one to two independent times with similar results.

Cell-based studies were performed at least three independent times with comparable results. AFU, arbitrary fluorescence units. Data represent mean ± SEM. Student's t test was used for statistical analysis: *$p < 0.05$; **$p < 0.1$.

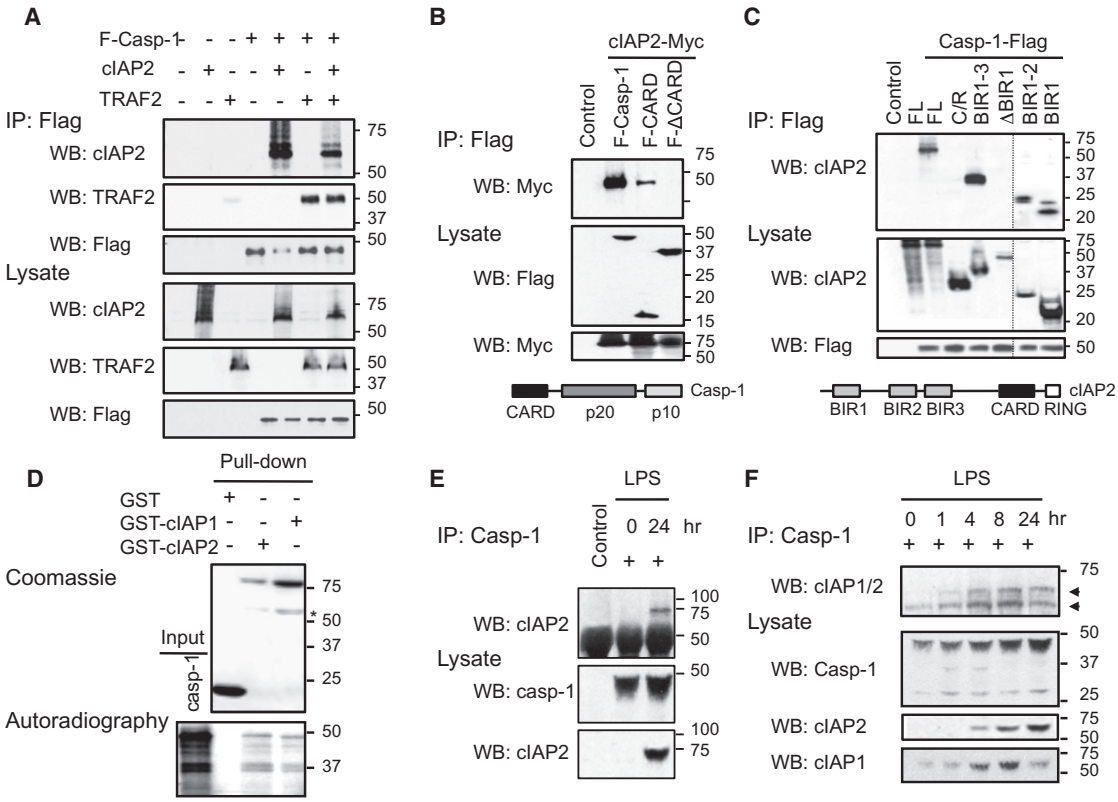

Figure 2. cIAP1, cIAP2, and TRAF2 Associate with Caspase-1-Containing Complexes

(A) HEK293T cells were transfected with Myc-cIAP2 or TRAF2 along with control vector or Flag-caspase-1. Caspase-1 was immunoprecipitated with M2-sepharose beads and coimmunoprecipitated cIAP2 and TRAF2 detected by immunoblotting as indicated.

(B) HEK293T cells were transfected with Myc-cIAP2 along with Flag-caspase-1 or a Flag-tagged caspase-1 deletion mutants. Caspase-1 was immunoprecipitated with M2-sepharose beads and coimmunoprecipitated cIAP2 detected by immunoblotting as indicated.

(C) HEK293T cells were transfected with HA-cIAP2 or a HA-tagged cIAP2 deletion mutants along with Flag-caspase-1. Caspase-1 was immunoprecipitated with M2-sepharose beads and coimmunoprecipitated cIAP2 detected by immunoblotting as indicated.

(D) In-vitro-transcribed and -translated caspase-1 labeled with ^{35}S-methionine (Input) was incubated overnight with bacterially produced GST, GST-cIAP1, or GST-cIAP2 bound to Sepharose beads (Pull down). Caspase-1 binding was revealed by autoradiography.

(E) THP-1 cells were stimulated with LPS (1 μg/ml) for 24 hr and endogenous caspase-1 immunoprecipitated with caspase-1 antibodies. cIAP2 coimmunoprecipitation was revealed by immunoblotting as indicated.

(F) THP-1 cells were stimulated with LPS (1 μg/ml) for different time points and endogenous caspase-1 immunoprecipitated with caspase-1 antibodies. cIAP1 and cIAP2 coimmunoprecipitation was revealed by immunoblotting as indicated.

All coimmunprecipitation experiments were performed independently two to three times with comparable results.

binding to these complexes (Figure 2B). Although CARD domains mediate homotypic interactions, the cIAP2 CARD was not required for its association with caspase-1 (Figure 2C). Rather, the N-terminal BIR1 domain of cIAP2 was necessary and sufficient for its interaction with caspase-1 (Figure 2C). To validate the association between caspase-1 and the cIAPs, we conducted in vitro pull-down experiments. Purified recombinant GST-cIAP1 or GST-cIAP2 fusion proteins, but not a GST control protein, specifically bound to ^{35}S-methionine-labeled in-vitro-transcribed and translated caspase-1 (Figure 2D). Furthermore, cIAP1 and cIAP2 interacted with caspase-1 at the endogenous level (Figures 2E and 2F). This was examined in LPS-stimulated THP-1 cells, because resting cellular amounts of cIAP2 are relatively low and expression is highly inducible by NF-κB (Wang et al., 1998). Indeed, cIAP2 was not detected in whole cell extracts of untreated cells, whereas it was strongly upregulated after LPS treatment and was found to coimmunoprecipitate with caspase-1 (Figures 2E and 2F). Altogether these results indicate an association of cIAP1, cIAP2, and TRAF2 with caspase-1-containing complexes and the requirement for these proteins for caspase-1 catalysis within the inflammasome.

The cIAP E3 Ligase Activity Mediates K63-Linked Polyubiquitination of Caspase-1

XIAP, cIAP1, and cIAP2 have all been reported to mono- or polyubiquitinate caspase-3, caspase-7, and caspase-9 (Choi et al., 2009; Huang et al., 2000; Morizane et al., 2005; Suzuki et al., 2001). To test whether the cIAPs could mediate caspase-1 ubiquitination, we expressed caspase-1, HA-ubiquitin, and cIAP2 in HEK293T cells, immunoprecipitated caspase-1 under highly stringent conditions, and probed for ubiquitin by immunoblot. At baseline, caspase-1 was weakly polyubiquitinated (Figure 3A, lane 2) but became markedly polyubiquitinated when cIAP2

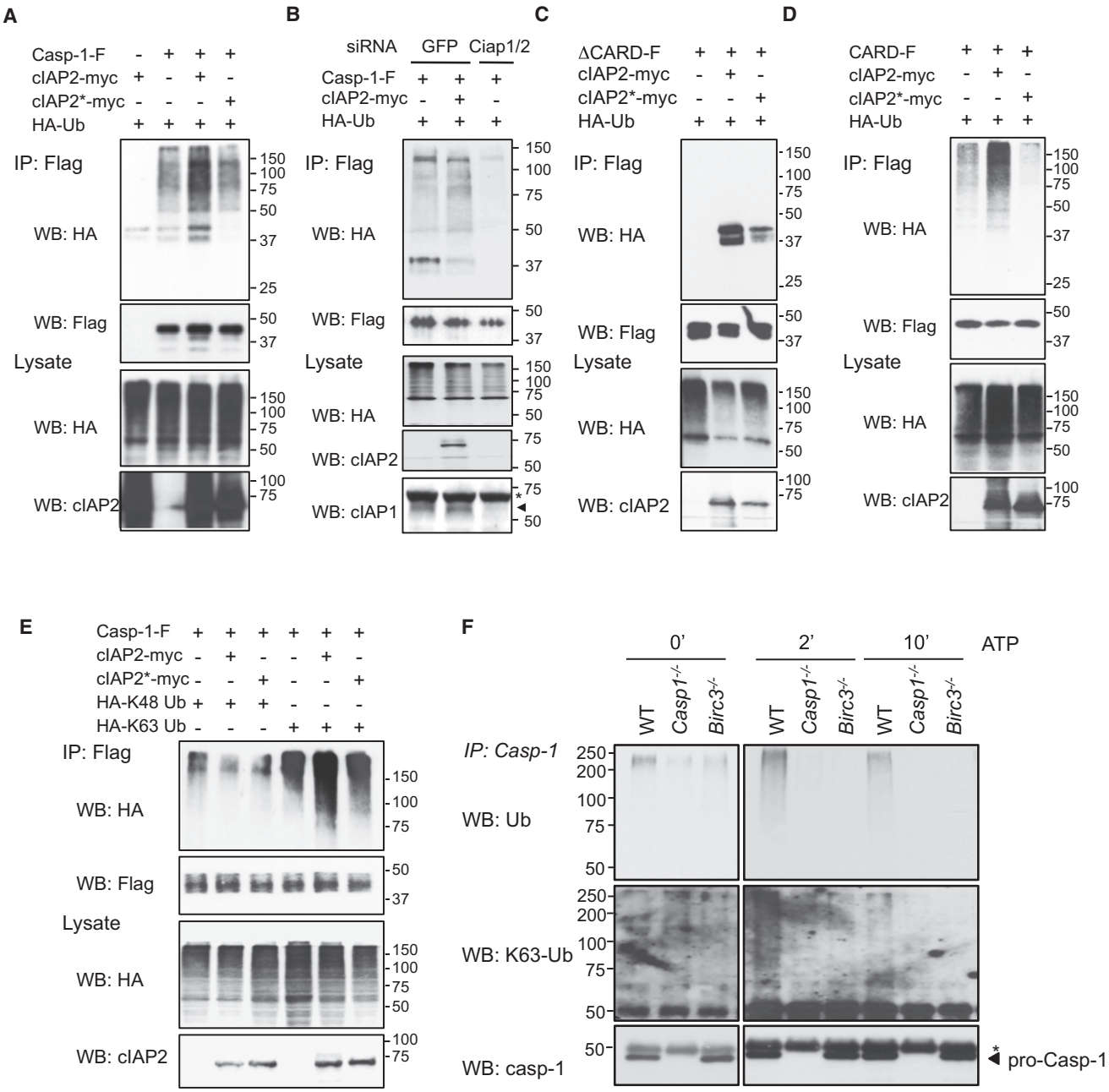

Figure 3. cIAPs Direct K63-Linked Polyubiquitination of Caspase-1

(A) HEK293T cells were transfected with HA-ubiquitin and Flag-caspase-1 along with Myc-cIAP2 or E3 inactive Myc-cIAP2*.

(B) HEK293T cells were transfected with siRNAs targeting cIAP1 and cIAP2 or GFP and subsequently transfected with Flag-caspase-1 and HA-ubiquitin.

(A and B) Caspase-1 was immunoprecipitated in high-stringency buffer and ubiquitinated caspase-1 detected by immunoblotting with HA antibodies.

(C) HEK293T cells were transfected with HA-ubiquitin and a Flag-tagged CARD-deletion caspase-1 mutant (ΔCARD-F) along with a Myc-cIAP2 or E3 inactive Myc-cIAP2*. Caspase-1-ΔCARD was immunoprecipitated in high-stringency buffer and ubiquitination detected by immunoblotting with HA antibodies.

(D) HEK293T cells were transfected with HA-ubiquitin and Flag-tagged CARD-only caspase-1 mutant (CARD-F) along with Myc-cIAP2 or E3 inactive Myc-cIAP2*. CARD-caspase-1 was immunoprecipitated in high-stringency buffer and ubiquitination detected by immunoblotting with HA antibodies.

(E) HEK293T cells were transfected with HA-tagged K48-only or K63-only ubiquitin in combination with Flag-caspase-1 and Myc-cIAP2 or E3 inactive Myc-cIAP2*. Caspase-1 was immunoprecipitated in high-stringency buffer and ubiquitination detected by immunoblotting with HA antibodies.

(F) BMDM from WT, *Birc3*$^{-/-}$, or *Casp1*$^{-/-}$ mice were primed with LPS (1 μg/ml for 4 hr) before treatment with ATP (5 mM). Cells were lysed in denaturing buffer, endogenous caspase-1 immunoprecipitated, and ubiquitination detected by immunobloting with antibodies against total ubiquitin or K63-specific chains.

All ubiquitination assays were performed independently two to three times with comparable results except the K63 immunoblot, which was performed once.

was expressed (Figure 3A, lane 3). Conversely, caspase-1 ubiquitination was markedly reduced with depletion of endogenous cIAP1 and cIAP2 (Figure 3B, lane 3). To determine whether the E3 ligase activity of cIAP2 was required for caspase-1 ubiquitination, we coexpressed caspase-1 with a catalytically inactive mutant of cIAP2 harboring an Ala in the position of the metal-coordination residue His^{574} (Cheung et al., 2008). This E3 inactive form of cIAP2 was impaired in mediating the polyubiquitination of caspase-1 (Figure 3A, lane 4). Casp-1-CARD, but not casp-1-ΔCARD, showed enhanced ubiquitination when coexpressed with cIAP2 (Figures 3C and 3D), suggesting that caspase-1 ubiquitination occurs through its ability to interact with cIAP2. To determine the type of ubiquitin chains catalyzed by cIAP2, we expressed caspase-1 with mutant forms of ubiquitin that can be only K48 or K63 linked and found that caspase-1 was mostly conjugated with K63-linked polyubiquitin chains (Figure 3E). Importantly, we confirmed the ability of cIAP2 to mediate caspase-1 polyubiquitination endogenously with wild-type, *Casp1*$^{-/-}$, and *Birc3*$^{-/-}$ BMDM. Treatment with an inflammasome agonist resulted in caspase-1 polyubiquitination in wild-type cells but not *Birc3*$^{-/-}$ macrophages (Figure 3F). Furthermore, probing with a linkage-specific antibody revealed that caspase-1 was modified with K63-linked polyubiquitin chains (Figure 3F). Together, our results show that the cIAP E3 ligase activity mediates nondegradative and signaling-associated K63-linked polyubiquitination of caspase-1.

The cIAPs Are Universal Regulators of Caspase-1 Activation

To investigate the physiological relevance of these findings, we examined IL-1β production by BMDM from wild-type, *Casp1*$^{-/-}$, or *Birc3*$^{-/-}$ mice in response to stimulation with various inflammasome agonists, including ATP, monosodium urate crystals (MSU), alum, *Salmonella typhimurium*, and cytosolic flagellin. In each case, IL-1β production was significantly reduced in *Birc3*$^{-/-}$ macrophages compared to wild-type cells, independently of an effect on pro-IL-1β or inflammasome component transcription (Figures 4A and 4B). Furthermore, the impaired production of mature IL-1β by *Birc3*$^{-/-}$ cells directly correlated with reduced caspase-1 activation and processing (Figure 4C). These results indicated that the cIAPs play an essential role in inflammasome activation, irrespective of the activating agonist or NLR engaged. Consistently, treatment of wild-type BMDM with SM resulted in dampened caspase-1 activation in response to extracellular ATP (Figures S2A and S2B). Conversely, prolonged SM treatment stimulated the inflammasome by triggering proinflammatory pathways that primed its activation (Figures S2A and S2B). SM alone had no effect on inflammasome activity (Figures S2A–S2C) but resulted in marked caspase-1 processing and IL-1β secretion in the presence of ATP alone (Figures S2B and S2C). This effect was abrogated by cycloheximide treatment and thus dependent on de novo protein synthesis. Immunoblotting analysis of pro-IL-1β levels in SM-treated macrophages indicated that prolonged SM exposure upregulated pro-IL-1β to levels similar to those obtained with LPS stimulation (Figure S2D). Importantly, genetic or SM-induced cIAP depletion had no effect on cell survival (Figures S3A and S3B). These results indicate that acute IAP depletion by SM treatment prevents inflammasome activation, but prolonged exposure to

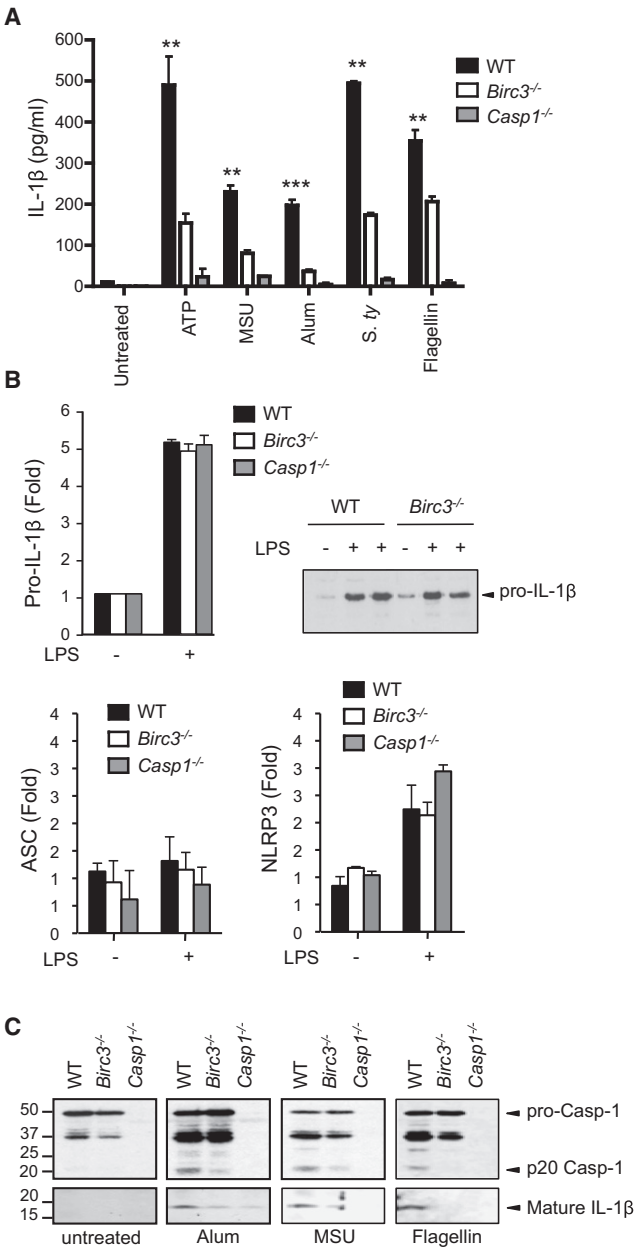

Figure 4. cIAP2 Is Required for Inflammasome Function in Macrophages

(A) BMDM from WT, *Birc3*$^{-/-}$, or *Casp1*$^{-/-}$ mice were primed with LPS (1 μg/ml for 4 hr) before treatment with the inflammasome agonists ATP (5 mM for 40 min), MSU (100 μg/ml for 5 hr), alum (100 μg/ml for 5 hr), liposome-encapsulated flagellin (2 μg/ml for 5 hr), or *S. typhymurium* (MOI 10 for 1 hr). IL-1β secretion in the supernatant was measured by ELISA.

(B) LPS-primed BMDM lysates were examined for pro-IL-1β expression by quantitative real-time PCR and immunoblot. ASC and NLRP3 expression were measured by quantitative real-time PCR.

(C) Lysates of cells treated as in (A) were analyzed by immunoblot for caspase-1 cleavage and IL-1β processing.

Inflammasome agonist assays were performed at least three independent times with similar results. Data represent mean ± SEM. Student's t test was used for statistical analysis: *$p < 0.05$; **$p < 0.1$.

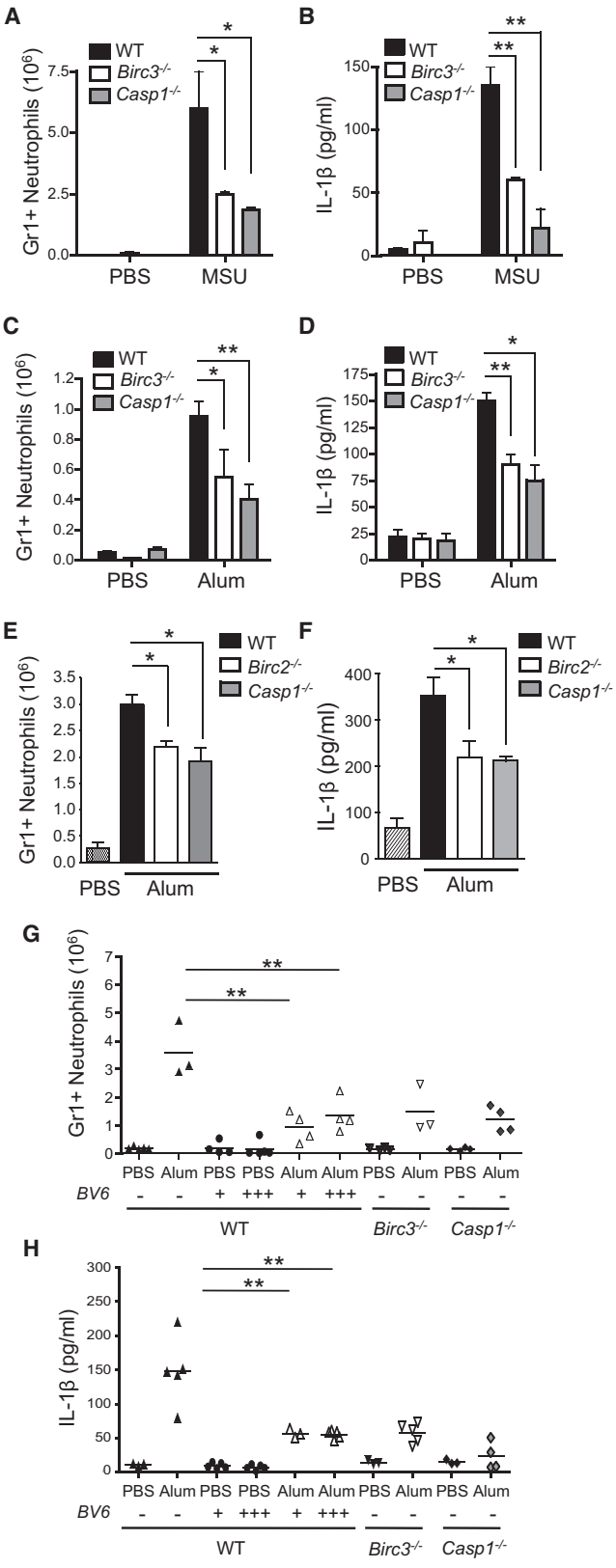

Figure 5. *Birc3*$^{-/-}$ Mice Exhibit a Blunted Inflammasome Response

(A) WT, *Birc3*$^{-/-}$, or *Casp1*$^{-/-}$ mice were injected intraperitoneally with MSU (1 mg/ml) or endotoxin-free PBS. GR-1^{+} cells in the peritoneal lavage were enumerated by flow cytometry 16 hr later.

(B) WT, *Birc3*$^{-/-}$, or *Casp1*$^{-/-}$ mice were injected intraperitoneally with MSU (1 mg/ml). IL-1β levels in the peritoneal lavage were quantified by ELISA 4 hr later.

(C) WT, *Birc3*$^{-/-}$, or *Casp1*$^{-/-}$ mice were injected intraperitoneally with alum (50 mg/ml) or endotoxin-free PBS. GR-1^{+} cells in the peritoneal lavage were enumerated by flow cytometry 16 hr later.

(D) WT, *Birc3*$^{-/-}$, or *Casp1*$^{-/-}$ mice were injected intraperitoneally with alum (2 mg/ml). IL-1β levels in the peritoneal lavage were quantified by ELISA 4 hr later.

(E and F) WT, *Birc2*$^{-/-}$, or *Casp1*$^{-/-}$ mice were treated as in (C) and (D), respectively.

(G) WT, *Birc3*$^{-/-}$, or *Casp1*$^{-/-}$ mice were treated as in (B). 2 hr prior to alum treatment, animals were injected intravenously with DMSO or BV6 (1 mg/kg, + ; or 3 mg/kg, +++).

(H) WT, *Birc3*$^{-/-}$, or *Casp1*$^{-/-}$ mice were treated as in (A). 2 hr prior to treatment, animals were injected intravenously with DMSO or BV6 (1 mg/kg, + ; or 3 mg/kg, +++).

Peritonitis experiments were performed two to three times on *Birc3*$^{-/-}$ mice and one time on *Birc3*$^{-/-}$ mice. BV6 experiments were performed once. n = 3–6 mice per experiment. Data represent mean ± SEM. Student's t test was used for statistical analysis: *p < 0.05; **p < 0.1.

these compounds might result in an indirect secondary inflammasome response.

cIAP-Deficient Mice Exhibit a Blunted Caspase-1-Dependent Inflammatory Response

To determine the contribution of the cIAPs to inflammasome-dependent signals in vivo, we examined the response of wild-type, *Casp1*$^{-/-}$, *Birc2*$^{-/-}$, or *Birc3*$^{-/-}$ mice to an acute peritonitis model, whereby intraperitoneal injection of inflammasome agonists such as alum or MSU triggers neutrophil infiltration in an IL-1-dependent manner (Hornung et al., 2008; Martinon et al., 2006). MSU injection led to a massive recruitment of GR-1^{+} cells to the peritoneum of wild-type mice (Figure 5A), but this response was significantly blunted in *Casp1*$^{-/-}$ or *Birc3*$^{-/-}$ mice (Figure 5A). Similarly, IL-1β production was induced in wild-type but not *Casp1*$^{-/-}$ or *Birc3*$^{-/-}$ mice (Figure 5B), indicating that impaired caspase-1 activation contributed to the blunted neutrophil recruitment phenotype. Similar results were obtained with alum-induced peritonitis in both *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ animals (Figures 5C–5F). Intravenous injection of wild-type mice with SM, 2 hr prior to alum administration, significantly reduced peritoneal GR-1^{+} cell recruitment (Figure 5G) and IL-1β production (Figure 5H) to levels measured in *Casp1*$^{-/-}$ animals, further supporting the requirement of the cIAPs in inflammasome activation in vivo. Notably, SM treatment alone did not trigger the synthesis of pro-IL-1β (Figure 5H) or that of other proinflammatory cytokines such as IL-6 (Figure S4). Thus, acute administration of SM conferred protection in vivo by repressing inflammasome signaling without any measurable secondary proinflammatory effects.

DISCUSSION

Inflammatory caspase activation by the inflammasome contributes to innate immunity by inducing cytokine production and

pyroptosis. The cIAPs are important regulators of both cell death and inflammation but their role in inflammatory caspase function has been unclear to date. Here, we demonstrate that cIAP1 and cIAP2 are essential promoters of caspase-1 activity and of the inflammasome innate immune pathway. Deficiency in either cIAP1 or cIAP2 severely impaired caspase-1 activation, resulting in a blunted inflammatory reaction in response to various inflammasome agonists. This has been demonstrated 3-fold, in a cell-free system, in primary macrophages, and in vivo. Indeed, the inflammatory response of *Birc3*$^{-/-}$ mice to systemic inflammasome agonist exposure is significantly blunted and equivalent to that of *Casp1*$^{-/-}$ mice. Together, these findings indicate that the cIAPs are integral to the inflammasome pathway.

The modalities of caspase-1 activation and regulation within the inflammasome are not fully understood. It has been suggested that caspase-1 catalysis occurs by induced proximity, in a manner analogous to caspase-9 activation by the apoptosome (Martinon et al., 2002), but whether additional modulators are required to amplify this response and induce maximal caspase-1 activation has not been examined. Our results indicate that the cIAPs are recruited to caspase-1-containing complexes and modulate their activity. We found that cIAP1 and cIAP2 physically interact with caspase-1. Binding occurs via the caspase-1 CARD and the first BIR domain of the cIAP. The IAP BIR domains are grouped according to the presence of a deep peptide-binding groove. In type 2 BIRs (BIR2 and BIR3), this hydrophobic cleft mediates binding to IAP-binding motif (IBM)-containing proteins such as SMAC and caspase-3, caspase-7, and caspase-9 (Scott et al., 2005; Srinivasula et al., 2001). Type 1 BIRs (BIR1), on the other hand, lack this groove and employ a different mode to interact with binding partners including inflammatory mediators such as TNFR-associated factors (TRAFs) or TGF-β-associated kinase binding protein (TAB1) (Lu et al., 2007; Samuel et al., 2006). Our results indicate that the cIAPs bind to caspase-1 through BIR1, which suggests a distinct nature of cIAP function in inflammatory versus apoptotic caspase regulation.

The inflammatory functions of the cIAPs are tightly associated with adaptor proteins of the TRAF family (Bertrand et al., 2008, 2009; Mao et al., 2010; Shu et al., 1996). Because the BIR1 of cIAP1 and cIAP2 constitutively binds the N-domain of TRAF2 as part of a preformed complex (Rothe et al., 1995; Shu et al., 1996), we examined a possible function for TRAF2 in caspase-1 activation. We found that TRAF2 is similarly associated with caspase-1-containing complexes and is required for its catalytic activity and downstream proinflammatory events.

Interestingly, the cIAP-caspase-1 interaction results in nondegradative K63-linked polyubiquitination of caspase-1. Apoptotic caspase ubiquitination by the IAPs is an evolutionarily conserved regulatory mechanism. In *D. melanogaster*, DIAP1 regulation of DRONC and drICE is dependent on polyubiquitination (Wilson et al., 2002). In mammalian cells, XIAP-mediated inactivation of apoptotic caspases requires a functional RING motif (Schile et al., 2008), and IAP-dependent mono- or polyubiquitinate caspase-3, caspase-7, and caspase-9 has been reported (Choi et al., 2009; Huang et al., 2000; Morizane et al., 2005; Suzuki et al., 2001), but the functional consequence of this modification has not been clearly defined. The ubiquitin ligase activity of the IAPs is also essential for their proinflammatory functions. Ubiquitination of IMD by DIAP2 permits NF-κB activation in *Drosophila* (Paquette et al., 2010), and the mammalian homologs of IMD, RIP1 and RIP2, are polyubiquitinated by cIAP1 and cIAP2 to activate NF-κB and MAP kinase signaling downstream of TNFR1 and NOD receptors, respectively (Bertrand et al., 2008, 2009). A previous study did not detect cIAP2-directed ubiquitination of caspase-1 in a reconstituted in vitro assay via recombinant E1, E2, and ubiquitin (Huang et al., 2000). Although this may indicate a requirement for cofactors absent in this minimal system, it is also possible that the E3 activity of the cIAPs functions to recruit ubiquitin binding domain-containing adaptor proteins and/or E3 ligases that are necessary for caspase-1 ubiquitination. Dissociating these two possibilities is a remaining challenge and a key step in understanding the functional significance of caspase-1 ubiquitination. Modification with nondegradative K63-linked polyubiquitin chains is an important scaffolding mechanism in innate immunity signaling (Bhoj and Chen, 2009). K63 ubiquitination of caspase-1 could serve a number of functions such as the recruitment and oligomerization of inflammasome components, the induction of conformational changes necessary for enhanced or sustained caspase-1 catalytic activity, or the engagement of additional proteins that mediate the stimulatory effects of cIAP1 and cIAP2 on caspase-1. The nature of the factor(s) recruited to caspase-1 through its polyubiquitin scaffolds is currently unknown and will require further investigation. Interestingly, unanchored K63 polyubiquitin chains have been recently shown to bind to and modulate the function of proinflammatory proteins including RIG-I (Zeng et al., 2010). Whether this is the case for caspase-1 or whether the K63 polyubiquitin chains are anchored to a specific lysine residue within the CARD of caspase-1 will also need to be examined.

The cIAPs are thought to be highly interdependent. They have been shown to exist in preformed heteromeric complexes, and changes in expression of one cIAP impact the stability of the other (Conte et al., 2006; Rajalingam et al., 2006). Previous studies from our group and others (Bertrand et al., 2008, 2009) have demonstrated that cIAP1 and cIAP2 function within a binary complex and play nonredundant roles in innate immunity. Consistently, we find that depletion of either cIAP1 or cIAP2 is sufficient to extinguish caspase-1 activation. Furthermore, although the role of XIAP in inflammasome function has not been thoroughly investigated, we found that, contrary to cIAP1 and cIAP2, XIAP expression had no effect on caspase-1 catalytic activity.

Altogether, our results identify cIAP1 and cIAP2 as critical mediators of inflammasome function. Although minimal conditions for inflammasome assembly do not include the cIAPs (Faustin et al., 2007), their activity greatly amplifies caspase-1 catalysis and their absence severely impairs inflammasome function under physiological conditions. Notably, pharmacological depletion of the cIAPs by SM treatment markedly inhibited caspase-1 activation both in vitro and in vivo. Several IAP antagonists are currently being developed as anticancer therapeutics, because several cancer cell lines have been shown to be sensitive to SM-induced apoptosis in vitro (Bertrand et al., 2008; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). Our in vivo results showing that acute SM administration inhibited the inflammasome response in an

experimental model of peritonitis provide support for the therapeutic potential of these compounds in inflammatory diseases. The use of SM must, however, be approached cautiously, because prolonged in vitro exposure leads to enhanced pro-IL-1β expression and maturation. This is possibly the result of spontaneous NIK-dependent TNF-α overproduction, which can serve as an efficient "priming" signal for the inflammasome (Bauernfeind et al., 2009). The absence of cIAP2 at basal conditions also further demonstrates the importance of the initial "priming" signal necessary for transcriptional upregulation of inflammasome components including cIAP2 itself.

The identification of the cIAPs as key regulators of the inflammasome pathway reveals insights into the mechanistic basis of caspase-1 activation and provides therapeutic opportunities for the treatment of immune-mediated inflammatory diseases.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

All cell culture supplies were from Wisent unless specified otherwise. Anti-human IL-1β was from Cell Signaling (cat# 2022) and anti-mouse IL-1β from R&D (cat# AF401NA). Anti-human caspase-1 p20 was obtained from Merck. Antibodies against the mouse caspase-1 p10 subunit were purchased from Santa Cruz (cat# sc-514) and antibodies against the p20 subunit were obtained from Genentech. Human cIAP1- and cIAP2-specific antibodies were purchased from R&D (cat# AF8181 and AF8171). Ubiquitin antibodies were purchased from Santa Cruz (cat# sc-8017) and anti-K63-specific antibodies from Millipore (cat# 05-1313). Anti-mouse-GR-1 with FITC tag was purchased from BD Biosciences (cat# 553126). Monoclonal antibodies for HA (cat# 11583816001) and Myc (cat# 11667149001) tag were from Roche. Anti-Flag monoclonal antibody (cat# F3165), M2 agarose beads (cat# A2220), and Flag peptide (cat# F3290) were from Sigma. Protein A (cat# P9424) and protein G (cat# P3296) sepharose beads were from Sigma. BV6 was obtained from Genentech. siRNA targeting human cIAP1 and cIAP2 were obtained from Invitrogen. siRNA directed against human TRAF2 (cat# J-005198-05) and GFP (cat# P-002048-01-02) were purchased from Dharmacon. Active recombinant human caspase-1 was purchased from BioMol (cat# SE-168). ATP was purchased from Sigma (cat# A3377), ImjectAlum was from Pierce (cat# PI77161), MSU was from Alexis Biochemicals (cat# ALX-400-047), Flagellin from Invivogen (cat# TLRL-STFLA), crude LPS from Sigma (cat# L2630), and ultra-pure LPS from Invivogen (cat# TLRL-PELPS). Lipofectamine 2000 was purchased from Invitrogen (cat# 11668-019) and DOTAP was from Roche (cat# 11202375001). RT-PCR reagents were all from Invitrogen and SYBR green was purchased from BioRad (cat# 1725852). DuoSet ELISA was purchased from R&D: anti-mouse-IL-1β (cat# DY401), anti-mouse-IL-6 (cat# DY406). For in vitro transcription and translation, a TNT T7 Rabbit Reticulocyte Lysate ITT kit was purchased from Promega (cat# PRL4610). LDH release was measured with the CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega (cat# G1780).

Animal Strains

Wild-type C57BL/6 mice were either bred in-house or purchased from a local supplier (Charles River Laboratories, St-Constant, Quebec). cIAP2- (*Birc3*$^{-/-}$) and caspase-1- (*Casp1*$^{-/-}$) deficient mice were on a pure C57BL/6 background and bred in-house at the McGill University animal housing facilities. All animal experiments were performed under guidelines of the animal ethics committee of McGill University.

Cell-Free Inflammasome Activation Assay

Cells were stimulated with 1 μg/ml crude LPS for 4 hr and, where indicated, 5 μM of the IAP antagonist BV6 for 1 hr. Cells were harvested and washed in cold PBS, followed by swelling in 5 volumes of ice-cold hypotonic buffer and mechanical disruption by passage through a 22G needle 15 times. The cytoplasmic fraction was harvested by the centrifugation and subsequently shifted to 30°C for 0, 5, 15, or 30 min to initiate the activation of caspase-1 and IL-1β cleavage in vitro. In cIAP2-depletion studies, supernatants were incubated on ice with sepharose beads coupled to protein-G-adsorbed polyclonal antibodies to cIAP2 for 1 hr prior to incubation at 30°C.

Primary BMDM Culture and Stimulation

The femurs and tibias of wild-type, *Birc3*$^{-/-}$, and *Casp1*$^{-/-}$ mice were flushed with cold RPMI with a 25G needle. The marrow plug was resuspended in RPMI and debris removed with a 75 μm filter. The cells were spun and resuspended in RPMI supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 μg/ml penicillin/streptomycin, and 20% L929 conditioned media, plated in non-tissue culture-treated dishes, and cultured at 37°C in 10% CO_2. Four days later, cells were split and allowed to grow for an additional 2 days before being plated for stimulation on day 6 of culture. For inflammasome agonist studies, cells were primed for 4 hr with 1 μg/ml ultra-pure LPS prior to stimulation with Opti-MEM minimal media (GIBCO) containing 5 mM ATP, 100 μg/ml MSU, or 500 μg/ml alum. Cells were transfected with 2 μg/ml flagellin (Invivogen) with DOTAP cationic lipid according to the manufacturer's instructions. The day of infection, a 3 mL overnight *S. typhimurium* (SL1344) culture was diluted 1:10 in LB broth and grown standing to logarithmic phase (OD_{600} = 0.9 = 5 × 10^8 cfu/mL). Bacterial pellets were washed extensively with cold PBS and diluted in Opti-MEM before infecting at MOI of 10. Supernatants and whole cell extracts were harvested 6 hr poststimulation or 1 hr postinfection.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from cells with the Trizol reagent followed by chloroform extraction and isopropanol precipitation. 0.5 μg of the resulting RNA was reverse-transcribed into cDNA by random hexamers and MML-V reverse-transcriptase in a total volume of 20 μl, according to the manufacturers' protocol. Quantitative PCR was performed with iTaq SYBR green supermix and primers directed against mouse pro-IL-1β: 5′-CGGCACACC CACCCTG-3′ (forward) and 5′-AAACCGCTTTTCCATCTTCTTCT-3′ (reverse); ASC: 5′-ACTTGTCAGGGGATGAACTC-3′ (forward) and 5′-TGGTACTGTCC TTCAGTCAG-3′ (reverse); NLRP3: 5′-CGAGACCTCTGGGAAAAAGCT-3′ (forward) and 5′-GCATACCATAGAGGAATGTGATGTACA-3′ (reverse) and housekeeping gene L32: 5′-GAAACTGGCGGAAACCCA-3′ (forward) and 5′-GGATCTGGCCCTTGAACCTT-3′ (reverse). Fold induction was calculated over untreated levels by the delta-delta-Ct method.

Intraperitoneal Injection of Inflammasome Agonists

For neutrophil recruitment assays, age- and sex-matched wild-type, *Birc2*$^{-/-}$, *Birc3*$^{-/-}$, and *Casp1*$^{-/-}$ mice were injected intraperitoneally with 1 mg MSU or 50 mg alum in 0.2 ml warm sterile PBS. Animals were euthanized by CO_2 overdose 16 hr after injection, and infiltrating cells were harvested by peritoneal lavage with 5 mL PBS, counted and labeled for 30 min in the dark with GR-1 antibodies coupled to FITC. The percentage of GR-1-positive cells was determined by FACSCalibur (BD Biosciences) flow cytometry. For IL-1β secretion studies, animals were injected as above with 1 mg MSU or 2 mg alum. The peritoneum was lavaged 4 hr postinjection with 1 ml cold PBS and IL-1β levels measured by ELISA. Where indicated, animals were injected intravenously with 200 ml PBS containing 1 mg/kg or 3 mg/kg BV6 or the corresponding concentration of DMSO 2 hr prior to intraperitoneal administration of alum.

Immunoprecipitation and Pull-Down Experiments

For overexpression and coimmunoprecipitation assays, HEK293T cells were transiently transfected with Flag-caspase-1 along with Myc-cIAP1, Myc-cIAP2, or TRAF2 via lipofectamine 2000. At 24 hr posttransfection, cells were washed in PBS, harvested, and lysed on ice for 30 min in B150 lysis buffer (20 mM Tris-HCl [pH 8.0], 150 mM KCl, 10% glycerol, 5 mM $MgCl_2$, 0.1% NP-40, and protease inhibitors) before performing three freeze-thaw cycles. For ubiquitination assays, cells were transfected along with HA-ubiquitin and lysed in RIPA buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, and protease inhibitor). Supernatants were incubated for 2 hr with M2 Flag-absorbed agarose beads at 4°C followed by three washes of the beads with the lysis buffer. Immunoprecipitates were eluted from the beads with Flag peptides. For endogenous coimmunoprecipitation studies, THP-1 cell lysates were incubated with

caspase-1 antibody and protein G sepharose beads. Immunoprecipitates were eluted by boiling in Laemmli buffer and processed for immunoblot analysis. For detection of endogenous ubiquitination BMDM from WT, *Birc3*$^{-/-}$, and *Casp-1*$^{-/-}$ mice were lysed in denaturing lysis buffer (1% SDS, 50 mM Tris [pH 7.4], 5 mM EDTA, 10 mM DTT, and protease inhibitor) then diluted in nondenaturing buffer (20 mM Tris, 140 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, protease inhibitors). Lysates were immunoprecipitated overnight with caspase-1 antibody and protein A sepharose beads. Immunoprecipitates were eluted by boiling in Laemmli buffer and processed for immunoblot analysis. For detection of total and K63-linked Ub, nitrocellulose membranes were denatured by autoclaving prior to immunodetection.

GST pull-down experiments were performed in NET-N buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 1% Triton X-100, protease inhibitor) with GST, GST-cIAP1, and GST-cIAP2 fusion proteins produced in bacteria from pGEX4T2 constructs.

Caspase-1 Fluorogenic Activity Assay and Propidium Iodide Staining

HEK293T cells were transiently transfected with caspase-1 in combination with pcDNA3.1, Myc-cIAP2, Myc-cIAP1, or Myc-XIAP and collected 24 hr later. For cleavage assays, cells were lysed in CHEGG buffer (50 mM HEPES/KOH [pH 7.0], 0.1% CHAPS, 2 mM Na-EDTA, 10% glycerol, 10 μl 1M DTT, and protease inhibitor) before sonicating for 15 s at 60% amplitude. Lysates were incubated with 10 μM of the fluorogenic caspase-1 substrate Ac-WEHD-AFC. The release of free AFC was monitored continuously for 1 hr (excitation 380 nm, emission 460 nm) in 1 min intervals and expressed as arbitrary fluorescence units per minute. For PI staining, cells were washed in PBS and resuspended in FACS buffer (2% FBS in PBS). Immediately prior to FACS analysis, PI was added to a final concentration of 0.5 μg/ml.

Statistical Analysis

Data are represented as average ± standard error. Two-tailed Student's t test was used for evaluating statistical significance between groups.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.immuni.2011.10.016.

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